Oxidative Stress in Toxicology: Established Mammalian and Emerging Piscine Model Systems

Sue A. Kelly,[1,2] Christine M. Havrilla,[2,3] Todd C. Brady,[2,4] Kimberly Harris Abramo,[2,3] and Edward D. Levin[2]

[1]Nicholas School of the Environment, [2]Integrated Toxicology Program, [3]Department of Chemistry, and [4]Department of Pathology, Duke University, Durham, NC 27708 USA

Interest in the toxicological aspects of oxidative stress has grown in recent years, and research has become increasingly focused on the mechanistic aspects of oxidative damage and cellular responses in biological systems. Toxic consequences of oxidative stress at the subcellular level include lipid peroxidation and oxidative damage to DNA and proteins. These effects are often used as end points in the study of oxidative stress. Typically, mammalian species have been used as models to study oxidative stress and to elucidate the mechanisms underlying cellular damage and response, largely because of the interest in human health issues surrounding oxidative stress. However, it is becoming apparent that oxidative stress also affects aquatic organisms exposed to environmental pollutants. Research in fish has demonstrated that mammalian and piscine systems exhibit similar toxicological and adaptive responses to oxidative stress. This suggests that piscine models, in addition to traditional mammalian models, may be useful for further understanding the mechanisms underlying the oxidative stress response. *Key words*: antioxidants, fish, lipid peroxidation, mammals, oxidative DNA damage, oxidative stress, reactive oxygen species. *Environ Health Perspect* 106:375–384 (1998). [Online 9 June 1998]
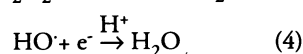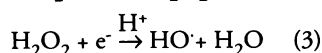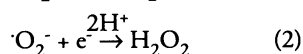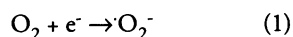http://ehpnet1.niehs.nih.gov/docs/1998/106p375-384kelly/abstract.html

The discovery of superoxide dismutase by McCord and Fridovich (1) provided the first evidence implicating radicals and oxidative stress as important sources of biological injury. Oxidative stress occurs when there is an imbalance in the generation and removal of radical species within an organism. The majority of these radicals involve oxygen and are referred to as reactive oxygen species (ROS) (2). Because of the potential for ROS to damage tissues and cellular components such as membranes (3,4), DNA (5–7), and proteins (8–10), oxidative stress has become a topic of significant interest in both mammalian and aquatic toxicology.

The majority of animal studies focusing on oxidative stress have employed mammalian cell or organ models to analyze the oxidative capacity of a particular toxicant or to evaluate the efficacy of a compound, such as an antioxidant, to protect from toxicant-mediated oxidative damage. Inflammation, ischemic reperfusion injury, aging, and carcinogenesis have all been linked to oxidative stress in mammalian systems (10–13). However, while the breadth of knowledge in mammalian systems is great, a considerable amount remains to be understood regarding cellular response mechanisms, disease etiology, and repair processes within cells. Notably, there is a growing literature on the effects of oxidative stress in aquatic organisms, and it is

becoming apparent that the general mechanisms of oxidative toxicity in both mammalian and piscine systems are similar, with comparable lesions and responses serving as markers of oxidative stress (14). Consequently, evidence suggests that fish have the potential to serve as suitable model organisms for studying the mechanistic aspects of oxidative toxicity, such as chemical carcinogenesis and damage repair, that are also relevant to biomedical toxicology.

Sources of Reactive Oxygen Species

Several basic cellular processes lead to the production of ROS within a cell. Cellular respiration involves the reduction of molecular oxygen (O_2) to water in the electron transport chain. This reduction occurs through four one-electron reductions resulting in the formation of reactive, partially reduced intermediates such as the superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO\cdot$) that may act as prooxidants:

$$O_2 + e^- \rightarrow \cdot O_2^- \quad (1)$$

$$\cdot O_2^- + e^- \xrightarrow{2H^+} H_2O_2 \quad (2)$$

$$H_2O_2 + e^- \xrightarrow{H^+} HO\cdot + H_2O \quad (3)$$

$$HO\cdot + e^- \xrightarrow{H^+} H_2O \quad (4)$$

One to 5% of these ROS may escape the electron transport chain and damage cellular components (15).

Several oxidizing enzymes produce ROS, comprising a second source of ROS within cells. Diamine oxidase, tryptophan dioxygenase, xanthine oxidase, and cytochrome P450 reductase can generate $\cdot O_2^-$, while enzymes such as guanyl cyclase and glucose oxidase generate H_2O_2 (16–18). Similarly, nitric oxide synthase produces $\cdot O_2^-$ under low arginine conditions (19). Other enzymatic processes produce extracellular $\cdot O_2^-$ and include the activity of the leukotriene generator lipoxygenase (20) and the prostaglandin generator cyclooxygenase (21). The enzymatic production of ROS is of additional interest in toxicology because some of these enzymes, such as cytochrome P450, are involved in the metabolism of xenobiotics (14,22).

Some radicals serve useful purposes. For instance, during the respiratory burst of leukocytes in immune-mediated host defense, monocytes, neutrophils, and macrophages produce $\cdot O_2^-$, nitric oxide ($\cdot NO$), and peroxynitrite ($ONOO^-$) (23). This response is triggered by stimulation of enzymes such as NADPH-oxidase (24), and these ROS are used as cytotoxic agents against pathogenic organisms (23). In addition to its use in the immune response, $\cdot NO$ can act as a neurotransmitter and muscle relaxant (25), while levels of other radicals play a role in the control of the transcription factors nuclear factor κB (NF-κB) and activator protein-1 (AP-1) (26). Additionally, superoxide has been proposed to terminate lipid peroxidation and may be mitogenic as well (27). The mitogenic function of $\cdot O_2^-$ is supported by a recent finding suggesting that $\cdot O_2^-$ may be a mitogenic signal in *ras*-mediated cellular transformation (28).

Xenobiotics comprise a third source of ROS because some xenobiotics can enhance the production of oxyradicals within cells. This may occur through processes such as inhibition of mitochondrial electron transport and subsequent accumulation of reduced intermediates (29), inactivation of antioxidant enzymes such as catalase (30,31), and depletion of radical scavengers (32–35). Of particular interest in toxicology are xenobiotics that are capable of redox cycling (Fig. 1). Quinones, some dyes, bipyridyl herbicides, some transition metals, and aromatic nitro compounds comprise classes of compounds known to redox cycle. Redox cycling involves the univalent reduction of the xenobiotic to a radical intermediate by enzymes such as xanthine oxidase and NADPH-cytochrome P450 reductase. This radical intermediate then transfers an electron to O_2, producing $·O_2^-$ and regenerating the parent compound. Thus, a single molecule of parent compound can generate many oxyradicals. Furthermore, this process occurs at the expense of cellular reducing equivalents, such as NADPH, which can have consequences for other metabolic processes (36–38).

Significance of Reactive Oxygen Species

Once produced, ROS may damage cellular components and tissues. Superoxide and HO· are two of the most studied ROS in this respect. Superoxide oxidizes catecholamines, tocopherols, ascorbate, and thiols (39,40). Superoxide can also inactivate enzymes such as catalase (31) and peroxidases (30). Furthermore, $·O_2^-$ inactivates [4Fe-4S]-containing dehydratases (e.g., aconitase, fumarase) and releases Fe^{2+} (41).

Much of the toxicity of $·O_2^-$ may be explained by its reaction with other ROS, such as H_2O_2, to form more reactive ROS. Of particular biological importance is the production and toxicity of HO· (Eq. 5 and 6). One of the most potent oxidants known, HO· reacts indiscriminately with cellular components such as lipids, DNA, and proteins. Because of its high reactivity, HO· has an in vivo lifetime of only a few nanoseconds (42). HO· may be produced by the reaction of $·O_2^-$ and H_2O_2 in the Haber-Weiss reaction (Eq. 6) (43).

$$2 ·O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$
$$·O_2^- + H_2O_2 \rightarrow HO· + OH^- + O_2 \quad (6)$$
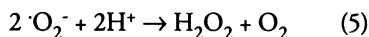
Because this reaction is kinetically slow (16), transition metals (e.g., iron and copper) often serve as catalysts. The iron-catalyzed Haber-Weiss reaction is depicted in Equations 7 and 8. Equation 8 is also known as the Fenton reaction (44).

$$·O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (7)$$

$$Fe^{2+} + H_2O_2 \rightarrow HO· + OH^- + Fe^{3+} \quad (8)$$
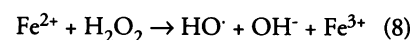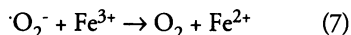
The polyanionic nature of cell membranes and DNA provides a structure for the adherence of metal cations. Thus, HO· produced in the Fenton reaction would be expected to occur adjacent to these critical biological targets, resulting in subsequent oxidation of lipids and DNA (45–47). The mechanistic aspects of oxidative damage to lipids and DNA are discussed below.

Lipid peroxidation. The important role of lipids in cellular components emphasizes the significance of understanding of the mechanisms and consequences of lipid peroxidation in biological systems. Polyunsaturated fatty acids (PUFAs) serve as excellent substrates for lipid peroxidation because of the presence of active bis-allylic methylene groups. The carbon–hydrogen bonds on these activated methylene units have lower bond dissociation energies, making these hydrogen atoms more easily abstracted in radical reactions (48). The susceptibility of a particular PUFA toward peroxidation increases with an increase in the number of unsaturated sites in the lipid chain (49).

Autoxidation of lipids in biological systems proceeds via a chain reaction consisting of three phases: initiation, propagation, and termination. Initiation of lipid peroxidation in vivo has not been extensively studied and is under much debate (50,51). This phase of lipid peroxidation may proceed by the reaction of an activated oxygen species such as singlet oxygen ($^1O_2$), $·O_2^-$, or HO· with a lipid substrate or by the breakdown of preexisting lipid hydroperoxides by transition metals. In the former case, peroxidation occurs by abstraction of a hydrogen atom from a methylene carbon in the lipid substrate (LH) to generate a highly reactive carbon-centered lipid radical (L·).

In the propagation phase of lipid peroxidation, molecular oxygen adds rapidly to
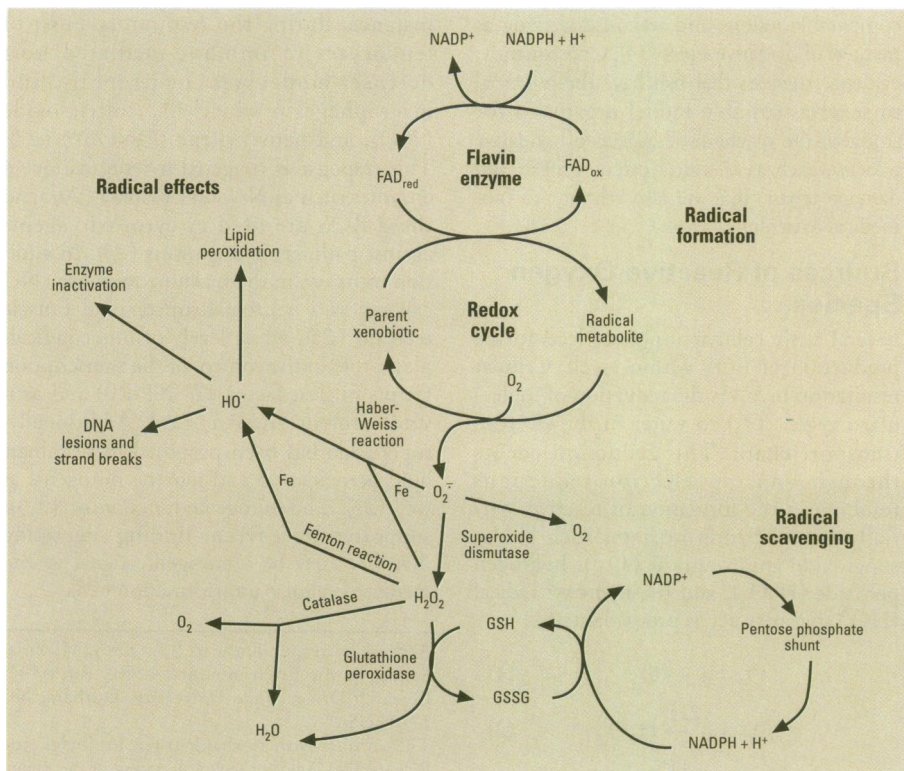
Figure 1. Redox cycling, which includes radical formation, scavenging, and effects. Abbreviations: GSH, glutathione; GSSG, glutathione disulfide. Adapted from Kappus (163).
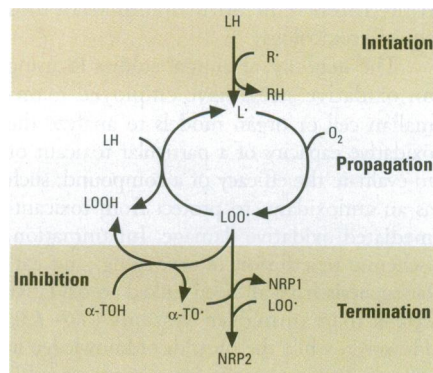
Figure 2. Overview of lipid peroxidation. Abbreviations: NRP, nonradical product; LOOH, lipid hydroperoxide; α-TOH, α-tocopherol; α-TO·, α-TOH radical; LH, lipid substrate; LOO·, lipid peroxyl radical. Adapted from Waldeck and Stocker (164).

L· at a diffusion controlled rate to produce the lipid peroxyl radical (LOO·) (4). The peroxyl radical can abstract a hydrogen atom from a number of *in vivo* sources, such as DNA and proteins, to form the primary oxidation product, a lipid hydroperoxide (LOOH). Alternatively, antioxidants such as α-tocopherol (α-TOH) can act as excellent hydrogen atom donors (52), generating LOOH and the relatively inert α-tocopherol phenoxyl radical (α-TO·). In the absence of antioxidants or other inhibitors, LOO· can abstract a hydrogen from another lipid molecule (LH), producing another highly reactive carbon centered radical (L·), which then propagates the radical chain (Fig. 2).

Transition metals are of particular interest to lipid peroxidation. In transition metal-catalyzed lipid peroxidation, HO· is thought to be the primary initiating radical species (51). However, both ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron, in addition to increasing the production of initiating hydroxyl radicals, can catalyze the propagation of the lipid peroxidation chain by decomposing LOOH oxidation products (53,54). The resulting alkoxyl (LO·) and peroxyl (LOO·) radicals are able to initiate new radical chains by interacting with additional lipid molecules. Other redox active metals such as copper ions may catalyze this reaction, but the availability of these ions under physiological conditions is yet unclear (23).

Termination of lipid peroxidation occurs via the coupling of any two radicals to form nonradical products. Nonradical products are stable and unable to propagate lipid peroxidation chains. Several extensive reviews of the chemistry of free radical oxidation of monoene and polyene unsaturated lipids have been discussed by Porter (55–57).

Repair of lipid peroxidation. Lipid peroxidation products modify the physical characteristics of biological membranes (58). For instance, incorporation of LOOH changes the physical structure of the membrane by decreasing the fluidity and increasing the permeability (59). Thus, the removal of the lipid peroxidation products from the membrane is necessary to repair the membrane damage (58) and is accomplished by two separate enzymatic systems: the sequential action of phospholipase A_2 with glutathione peroxidase (60) and phospholipid hydroperoxide glutathione peroxidase (61).

Phospholipases are activated by lipid peroxidation (62). Phospholipase A_2 (PLA_2) shows a substrate specificity for peroxidized phospholipids in membranes, catalyzing the hydrolysis of the phospholipid hydroperoxides to the hydroperoxy fatty acids (63–65).

Once released, the fatty acid hydroperoxides may undergo a reaction with glutathione peroxidase (GPx) to form stable, reduced hydroxy products (58,66). This terminates lipid peroxidation. A second enzymatic system eliminates phospholipid hydroperoxides from lipid membranes through the direct reaction of phospholipid hydroperoxide glutathione peroxidase with the esterified phospholipid hydroperoxides. This *in situ* reduction of the phospholipid hydroperoxides to phospholipid hydroxides while still incorporated in the membrane structure also results in the halt of membrane lipid peroxidation (67).

Detection of lipid peroxidation. Because direct analysis of endogenous primary lipid peroxidation products is complicated, the extent of lipid peroxidation is typically assessed by measuring levels of secondary oxidation products. The primary lipid oxidation products, LOOH, are unstable and decompose to form secondary products such as aldehydes and ketones through a multitude of reaction pathways (68). The resulting diverse array of breakdown products, coupled with the small *in vivo* concentration of these products, presents a challenge for accurate quantification of lipid peroxidation. The most used assay for lipid peroxidation is the thiobarbituric acid reactive substances (TBARS) test. Aldehydic compounds are reactive and highly cytotoxic (69–71), and the TBARS test relies on the production of a colored adduct from the reaction of lipid peroxidation products and thiobarbituric acid (72). This test is most frequently used as a measure of malondialdehyde (MDA), a secondary lipid oxidation product. However, because thiobarbituric acid reacts with a number of other oxidation products including 4-hydroxynoneal, other unsaturated aldehydes (73), and endoperoxides from enzymatic routes (74), this test is nonspecific (75). Therefore, the TBARS test can only give a crude measure of lipid peroxidation. Consequently, as an assay for MDA, the TBARS test generally gives higher values for TBARS reactivity compared to more rigorous tests for MDA (76,77).

Another crude measure of lipid peroxidation is the determination of diene conjugation. Generation of lipid peroxides typically involves the formation of conjugated dienes from the PUFA substrate. These conjugated hydroperoxide products absorb UV light in the region of 230–235 nm. However, many other endogenous materials also absorb UV light in the region of interest, creating a high background absorbence. Thus, this analytical technique is relatively insensitive to small changes in product formation (78). While these crude methods of measuring lipid peroxidation may be less

sensitive, they are often adequate for the question at hand and are more easily performed than more rigorous alternatives.

Oxyradical-generated DNA damage. DNA is another key cellular component that is particularly susceptible to oxidative damage. The primary oxidant responsible for DNA damage is HO·, as neither H_2O_2 nor peroxyl radicals react directly with DNA (79). The heterogeneity of DNA allows for many susceptible sites for HO· attack, including the nitrogenous bases and the sugar–phosphate backbone. The rate of HO· reaction with bases is approximately five times greater than that with the backbone (80). Lesion production for both portions of the DNA structure have been thoroughly reviewed (6,7).

Generally speaking, HO· attack on DNA bases leads to three classes of damage: hydroxylation, ring opening, and fragmentation. The resulting lesions are usually products of the secondary reactions that occur after the initial radical attack. Although over 100 different DNA lesions may result from HO· attack on DNA bases (81), the lesions most commonly used as biomarkers are 8-hydroxy-2′-deoxyguanosine (8-OHdG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). Both 8-OHdG and FapyGua result from HO· attack on deoxyguanosine, and the differentiating step between them is a secondary reaction. The potential steps of formation for both biomarkers are illustrated in Figure 3. These lesions may be directly detected in the DNA or assayed in urine. Specific analytical methods that detect biomarkers of oxidative damage to DNA bases have been reviewed (82).

In addition to producing direct damage to bases, a transient radical species generated within the DNA base may covalently bind with other macromolecules within the cell, forming intermolecular complexes. Perhaps the most important example of this phenomena is the protein–DNA crosslink. While the formation of such crosslinks has been reviewed (83), little is known about the significance of the different classes and structures (84).

Hydroxyl radicals may also attack the sugar–phosphate backbone of DNA, causing a different variety of lesions, including base free sites or apurinic sites (AP sites) where the base has been removed by oxidant-mediated reactions. An indicator of such damage is the presence of free bases in urine. Another indicator for radical attack on the DNA backbone is the fragmentation of deoxyribose. Single strand breaks occur via hydrogen abstraction at the C-4 position, leading to oxidation of the sugar moiety. This may be coupled with a second sugar oxidation on

the complimentary strand, causing a double strand break. Strand breaks in the DNA molecule may prove mutagenic or even lethal for the cell. (79).

Repair of oxidative DNA damage. There are a variety of cellular defense mechanisms that are designed to protect the genome from radical attack or repair oxidative damage. Some of these defenses include compartmentalization of the sensitive target molecules (81) and shielding of nonreplicating DNA by histones and polyamines (6). If protection of the genetic material is not successful, cellular regulatory mechanisms such as apoptotic induction and inhibition of cell cycle progression may prevent transfer of genetic damage to daughter cells (81). Alternatively, DNA repair processes may

correct the damage. Excision repair is performed by enzymes such as DNA glycosylases and AP endonucleases and occurs before replication, while postreplication repair provides a method for repairing lesions during or after the replication process. Both of these processes are believed to be constitutive and inducible (85). Unlike the repair of damaged bases, many of the processes that are involved in repair of strand breaks are not fully understood (81).

Antioxidant Defenses in Eukaryotes

To prevent damage to cellular components, there are numerous enzymatic antioxidant defenses designed to scavenge ROS in the cell. Superoxide dismutases (SODs) are

enzymes that scavenge $\cdot O_2^-$ by a rapid dismutation reaction ($k > 10^9$ $M^{-1}sec^{-1}$) (1):

$$2 \cdot O_2^- + 2 H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (9)$$
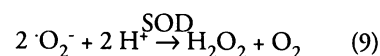
There are three SOD isoforms in eukaryotes: manganese SOD (Mn-SOD), copper/zinc SOD (Cu/Zn-SOD), and extracellular SOD (EC-SOD) (41). Mn-SOD, which contains a manganese prosthetic group, resides in the mitochondria, perhaps because of the need to protect mitochondrial proteins, membranes, and DNA from $\cdot O_2^-$ generated as a result of the respiratory chain. The Cu/Zn-SOD, containing copper and zinc prosthetic groups, is a cytosolic SOD. EC-SOD, also containing copper and zinc prosthetic groups, is secreted and binds to the plasma membrane and heparin-containing elements of the extracellular matrix (86). All forms of SODs are thought to dismutate $\cdot O_2^-$ via a ping-pong mechanism whereby the transition metal prosthetic group is reduced by $\cdot O_2^-$, forming O_2. The metal in the prosthetic group is then immediately re-oxidized by another $\cdot O_2^-$ molecule, resulting in the production of H_2O_2 (17).

As mentioned previously, the production of H_2O_2 within the cell may lead to the production of HO$\cdot$ and subsequent cellular damage via the metal-catalyzed Haber-Weiss reaction. Thus, it is important to remove H_2O_2. Catalase, a manganese or heme-containing enzyme, functions to rapidly dismutate H_2O_2 to water and oxygen ($k > 10^7$ M^{-1} sec^{-1}) (87):

$$2 H_2O_2 \xrightarrow{CAT} 2 H_2O + O_2 \quad (10)$$
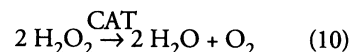
Catalase is mainly found in peroxisomes, perhaps because of the large number of H_2O_2-producing oxidases found in these organelles, while lower levels are also found in mitochondria and the cytosol. Several reports suggest that catalase can metabolize alkyl peroxides, such as methyl hydroperoxide and ethyl hydroperoxide in addition to H_2O_2 (88), but these events are thought to be an insignificant component of the activity of the enzyme (89).

A third enzymatic antioxidant, GPx, catalyzes the reduction of organic peroxides (ROOH), which are potential radical forming species within the cell. Glutathione (GSH), a nonenzymatic antioxidant, provides the reducing equivalents needed by GPx to carry out the reduction of organic peroxides to the corresponding alcohols and water:
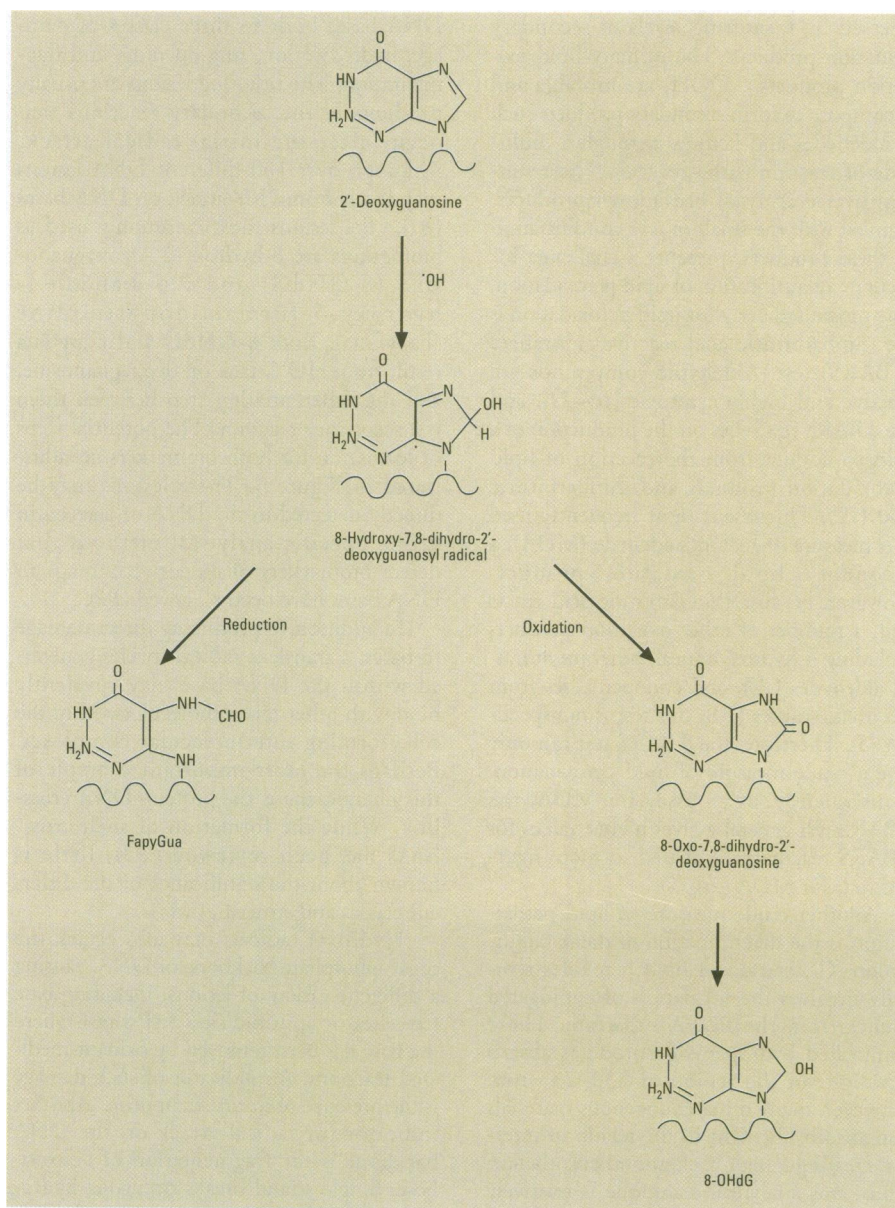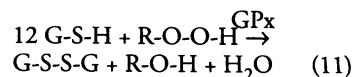
Figure 3. Mechanisms of formation of the common DNA lesions 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 8-hydroxy-2′-deoxyguanosine (8-OHdG). Adapted from Cadet (165).

$$12 \text{ G-S-H} + \text{R-O-O-H} \xrightarrow{GPx}$$
$$\text{G-S-S-G} + \text{R-O-H} + H_2O \quad (11)$$

There are several cytosolic, selenium-containing GPxs as well as an extracellular form. These enzymes also have high affinity for H_2O_2 and may be important for maintenance of low intracellular levels of H_2O_2 in the cytosol (88), where catalase levels are low. An enzyme important to the function of GPx is glutathione reductase (GR), which reduces oxidized glutathione (GSSG, glutathione disulfide) in the presence of NADPH in order to regenerate levels of GSH (90,91):

$$G\text{-}S\text{-}S\text{-}G + NADPH + H^+ \xrightarrow{GR}$$
$$2\ G\text{-}S\text{-}H + NADP^+ \qquad (12)$$
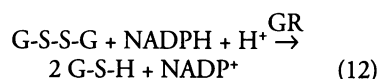
Levels of antioxidant enzymes, including SODs, catalases, GPx, and GR, are low in the extracellular space (50,92). Similarly, plasma levels of SODs and catalases are particularly low when compared to intracellular levels (92). Thus, a diverse array of small molecule, nonenzymatic antioxidants complete an efficient network of intra- and extracellular defenses necessary to modulate the levels of ROS. Nonenzymatic antioxidants may be classified in terms of their location and mode of action. Some small molecule antioxidants can act by scavenging oxidants or chelating transition metal ions (93).

Lipid soluble membrane-bound antioxidants are efficient in preventing oxidative damage to biomolecules. Vitamin E is an important lipid soluble antioxidant present in cells, as it is the major chain terminating antioxidant in biological membranes (94) and scavenges a wide array of ROS including $^1O_2$, $HO\cdot$, $\cdot O_2^-$, peroxyl, and alkoxyl radicals. Vitamin E is composed of a number of derivatives of tocopherols and tocotrienols. The major isomer in humans is α-TOH, which also possesses the greatest antioxidant activity of any vitamin E derivative. In homogeneous solutions, α-TOH is a strong inhibitor of polyunsaturated lipid peroxidation (52) and in vivo, most of the cellular vitamin E is concentrated in the lipid membranes (95,96). The primary antioxidant activity of tocopherols is to stop chain propagation of peroxyl radicals ($k = 6 \times 10^3 - 3 \times 10^6\ M^{-1}\ sec^{-1}$) (97). Tocopherols can typically scavenge two ROS per tocopherol molecule (98).

Other lipid soluble antioxidants contribute to the protection of lipid bilayers from oxidative stress. Ubiquinol Q_{10}, which plays an important role in mitochondrial electron transport (99), acts as a potent antioxidant in lipoproteins (92) and other lipid membranes (100). Ubiquinols are redox active quinone derivatives with a hydrophobic isoprenyl tail. They can rapidly react with oxygen, peroxyl, and alkoxyl radicals, preventing initiation of biomolecule damage. Ubiquinol can also

directly interact with α-TO$\cdot$, regenerating active α-TOH (101). Carotenoids are also lipid soluble antioxidants that are present in lipid membranes. Two of the main carotenoids, α- and β-carotene, serve as provitamin A analogues and are effective antioxidants as $^1O_2$ quenchers and peroxyl radical scavengers (102).

Ascorbic acid (vitamin C), a water soluble antioxidant, exists primarily as ascorbate at physiological pH. Ascorbate is a powerful reducing agent capable of rapidly scavenging a number of ROS including $\cdot O_2^-$ ($k = 2.7 \times 10^5\ M^{-1}sec^{-1}$) (103,104). In addition, ascorbate reacts with other cellular prooxidants such as $^1O_2$, hypochlorous acid (HOCl), and thiol radicals (RS$\cdot$) (103,105), Ascorbate is also able to reduce the vitamin E derived α-TO$\cdot$. Through this mechanism, ascorbate in the aqueous phase is able to regenerate membrane-bound α-TOH, prolonging the lifetime of this important antioxidant in the lipid phase and effecting removal of the radical from the lipid to the aqueous phase. In its action as an antioxidant, ascorbate is rapidly oxidized to dehydroascorbate, which shows little antioxidant activity. Dehydroascorbate may be reduced back to ascorbate via a GSH-dependent reductase (106).

In addition to acting as a reducing agent, GSH also provides antioxidant protection in the aqueous phase of cellular systems. GSH is synthesized in several different cell types and is present intracellularly in millimolar concentrations, making it the most abundant thiol with levels up to 10 mM (90). The cysteine thiol moiety of GSH imparts the antioxidant activity of this molecule. Like ascorbate, GSH can directly reduce a number reactive oxygen species including $^1O_2$, $HO\cdot$, and $\cdot O_2^-$, and GSH is oxidized to GSSG in this process. Relatively high ratios of GSH/GSSG are maintained intracellularly through the action of GR in an NADPH-dependent reaction (90,91). As mentioned previously, GSH also acts as a substrate or cosubstrate in many essential enzymatic reactions, such as with the antioxidant enzyme GPx. Thus, the depletion of GSH during oxidative stress could have a significant impact on the antioxidant poise within a cell.

It should be noted that antioxidants also have the potential to act as prooxidants under certain conditions. For example, ascorbate, in the presence of a high concentration of ferric iron (Fe^{3+}), is a potent potentiator of lipid peroxidation. This behavior results from the reduction of Fe^{3+} to Fe^{2+} by the strong reducing agent ascorbate (107), enabling redox cycling of the iron. Other antioxidants may behave similarly; Okada et al. (108) demonstrated the physiologic relevance of such reactions in the proximal tubule of ddY mice exposed

to Fe^{3+}-nitrilotriacetate. The release of cysteine during the hydrolysis of GSH in the tubule, followed by the reduction of Fe^{3+} by this cysteine, appears to lead to lipid peroxidation in the proximal tubule.

Piscine Systems as Models for Oxidative Stress Studies

Typically, mammalian species have been used as models to study oxidative stress and to elucidate the mechanisms behind cellular damage and response, largely because of the interest in human health issues surrounding oxidative stress. However, the aquatic environment provides a sink for many environmental contaminants that have the potential to cause oxidative stress in aquatic organisms. Some common pollutants in this category include polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), quinones, aromatic nitro compounds, aromatic hydroxylamines, bipyridyls, and transition metals (14). These compounds may cause adverse effects in aquatic organisms (e.g., lipid and DNA damage). Thus, a better understanding of oxidative stress in aquatic organisms is essential to understanding the impacts of these pollutants on aquatic systems.

However, with the growing interest in oxidative toxicity, the potential benefits of using fish as model organisms to study oxidative stress reach beyond applications to aquatic ecotoxicology. The similarities in the types of oxidative damage and defenses between mammalian and piscine systems suggest that fish can provide model systems for further understanding how ROS damage cellular components, how cells respond to and repair this damage, and how oxidative damage can lead to disease. This type of information could lead to the development of more sensitive markers for exposure and toxicity, and these markers may prove invaluable for evaluating subtle effects of oxidative stress, such as genotoxicity (35), in both mammalian and aquatic systems. Additionally, fish may provide an alternative system to rodents for evaluating the link between exposure and effect for diseases related to oxidative toxicity. For example, in some populations of aquatic organisms, the link between neoplasia and environmental pollution is more obvious than that in mammalian studies (109). Combined with other benefits, such as the potential for decreased cost, the ability to directly observe embryonic development in some fish species, and the advent of transgenic fish, piscine systems have the potential to provide suitable and beneficial models for studying oxidative stress in vertebrate systems and may help to bridge the gap between ecological and human health issues in oxidative toxicology.

Lipid peroxidation and DNA damage as indicators of oxidative stress. In both mammalian and piscine systems, oxidative stress causes toxic and adaptive responses within a cell (*14*). Toxic responses include damage to cellular components such as lipid peroxidation and DNA damage, as described earlier. In mammalian systems, transition metals are a classic source of radical generation and subsequent cellular damage. Lipid peroxidation has been used as a measure of this xenobiotic-induced oxidative stress. For example, iron and/or copper have been shown to induce lipid peroxidation in rat hepatocytes (*110*), rat testes (*111*), macrophages (*112*), and rat liver mitochondria (*113*).

Lipid peroxidation has also been used as an indicator of oxidative damage in fish. In a study of Atlantic croaker (*Micropogonias undulatus*), cadmium exposure resulted in an increase in MDA levels in homogenates of hepatic and ovarian tissues and in microsomes. Similarly, Aroclor 1254 caused an increase in MDA in croaker liver *in vivo* and *in vitro*, as well as in ovarian homogenates (*32*). Parihar and Dubey (*114*) found that freshwater Indian catfish (*Heteropneustes fossilis*) exposed to a temperature increase (from 25 to 37°C) exhibited significant increases in MDA levels in gill and air sac tissues. A different study in channel catfish (*Ictalurus punctatus*) evaluated lipid peroxidation following exposure to sediments contaminated with aromatic hydrocarbons. At all time points evaluated, the concentration of MDA in hepatic tissue was significantly elevated in fish exposed to contaminated sediments as compared to fish exposed to noncontaminated sediments (*115*).

Oxidative damage to DNA is another consequence of oxidative stress that has been used as a marker of this phenomenon. There are numerous recent reports in the literature indicating that iron exposure leads to DNA damage via oxidative mechanisms in mammalian systems. Examples of such studies include DNA–protein cross-links in a murine cell line (*116*), as well as 8-OHdG in the rat testis (*111*) and human pulmonary epithelia after exposure to asbestos (*117*). The iron-associating properties of asbestos are also related to double-strand DNA breaks in pulmonary epithelia (*118*), and double-strand breaks caused by iron complexed with certain chelators, such as 8-hydroxyquinolone, have been demonstrated in human lung (*119*). Similarly, copper causes elevated levels of 8-OHdG in rat liver and kidney (*120*), DNA–protein cross-linking in human lymphocytes (*121*), and increased levels of 8-OHdG in murine keratinocytes exposed to copper and benzoyl peroxide (*122*).

As with lipid peroxidation, oxidative damage to DNA has received increasing attention in aquatic organisms in recent years. For example, juvenile English sole (*Parophrys vetulus*) treated with nitrofurantoin, a known redox cycling xenobiotic, demonstrated an increase in hepatic 8-OHdG (*123*). Another DNA lesion linked to oxidative stress and recently found in fish is FapyGua. Malins et al. (*124*) found the FapyGua lesion in the DNA of neoplastic hepatic tissues of feral fish (English sole) but not in the nonneoplastic tissues. A similar study of feral English sole also found that fish from sites contaminated with PAHs and PCBs showed a high degree of hepatic DNA damage in the form of 8-OH adducts of guanine and adenine as well as FapyGua (*125*). These studies on lipid peroxidation and oxidative DNA damage demonstrate that mammals and fish exhibit similar lesions and that the markers used to detect toxic consequences of oxidative stress in mammalian systems are also useful in piscine models.

Cellular defenses. Fish exhibit many of the same defenses against oxidative stress as do mammals, suggesting that fish and mammalian systems have similar cellular responses to oxidative stress. These defenses include both low molecular weight free radical scavengers such as GSH, α-TOH, and ascorbic acid, as well as enzymatic defenses such as SOD, catalase, and GPx (*35,126*). Many studies in the aquatic literature provide baseline information for comparative purposes and have been reviewed by Winston and Di Giulio (*35*). It is clear from these studies that there is a significant degree of variation in the basal activities of antioxidant defenses between fish species, and much of this work has focused on enzymatic defenses. In comparison to other vertebrate systems, fish appear to exhibit lower basal activities of SOD and catalase, but have increased activities of GPx (*127–131*) and this should be taken into consideration when designing studies with fish.

Several studies in mammals and fish have demonstrated the importance of antioxidant defenses in protecting cells and organisms from oxidative damage and toxicity. For instance, tocopherol diminished lipid peroxidation in rat hepatocytes treated with iron and copper (*110*) and in rat lung and liver treated with iron (*132*), while both tocopherol and β-carotene were effective at reducing hepatic lipid peroxidation in rats fed high iron diets (*133*). In aquatic systems, studies have centered more on the effects of depleting antioxidant defenses rather than the effects of supplementation as seen in mammalian studies. For instance, a study of blue gill sunfish BF-2 fibroblasts

showed that depletion of GSH by agents such as buthionine sulfoximine, maleic acid, or 1-chloro-2,4-dinitrobenzene increased the toxicity of H_2O_2 and nitrofurantoin to fibroblasts. Furthermore, inhibition of SOD with diethyldithiocarbamate also enhanced the toxicity of H_2O_2 and paraquat (*134*). In freshwater Indian catfish, a decline in levels of ascorbic acid following temperature stress was correlated with an increase in lipid peroxidation in gill and air sac membranes, suggesting that ascorbic acid plays a protective role in preventing lipid peroxidation (*114*), perhaps through regeneration of tocopherol. Williams et al. (*135*) examined the importance of vitamin E in protecting rainbow trout (*Oncorhynchus mykiss*) from lipid peroxidation. Feeding trout a vitamin E-deficient diet resulted in an 18-fold decrease in liver microsomal levels of vitamin E and a significant increase in susceptibility to Fe^{3+}-ascorbate dependent lipid peroxidiation (measured as MDA) in liver microsomes. Additionally, vitamin E depletion also enhanced the amount of lipid hydroperoxides generated *in vivo* as seen in extracts of microsomes and plasma (*135*).

In addition to providing protection in both mammalian and piscine systems, some of the low molecular weight ROS scavengers and enzymatic antioxidant defenses are responsive to challenge with compounds that cause oxidative stress (i.e., they are induced), representing an adaptive consequence of exposure. In mammals, numerous studies have demonstrated that antioxidant enzymes are up regulated, particularly in response to toxicant-induced inflammation. The most studied of these enzymes is Mn-SOD, the activity and transcription of which is increased following exposure to toxins such as asbestos (*136*) and lipopolysaccharide (*137*), as well as inflammatory cytokines such as TNF-α (*136*). Similarly, phagocytosis of particulate matter leads to increased SOD activity in macrophages (*138*). Cu/Zn-SOD, EC-SOD, and glutathione-related enzymes are less commonly up regulated following toxic exposures, but lipopolysaccharide has been shown to induce transcription of both GPx and Cu/Zn-SOD (*139*), while toxicants that induce the inflammatory cytokine IFN-γ may up regulate transcription and secretion of EC-SOD (*140,141*).

Similar responses have been reported in aquatic species, and these may serve as biomarkers of exposure for certain compounds in aquatic organisms (*14*). For instance, Pedrajas et al. (*142*) found that sea bream (*Sparus aurata*) treated with copper chloride demonstrated increased SOD activity. Otto and Moon (*143*) found that PCBs altered

antioxidant defenses in trout by demonstrating that trout treated with 3,3',4,4'-tetrachlorobiphenyl showed increased GPx, GR, SOD, and catalase activities as well as increases in GSH and GSSG.

Notably, sometimes reductions are seen in the levels of antioxidants with or without an increase in others. For example, in a study of channel catfish exposed to bleached kraft mill effluent, the fish exhibited increased hepatic catalase activity and decreased hepatic GSH (33). Similarly, Thomas and Wofford (32) showed that Atlantic croaker exposed to cadmium exhibited a decrease in GPx activity and ascorbic acid content in hepatic and ovarian tissues while croaker in the same study exposed to Aroclor 1254 showed an increase in GPx activity in hepatic and ovarian tissues without any change in ascorbic acid content of either tissue. While not necessarily an adaptive response, a decrease in levels of antioxidant defenses is biologically significant and can also serve as a marker of oxidative stress. Combined with the presence of antioxidant inductions, it may be possible to develop a response profile indicative of some xenobiotics.

Field studies. In addition to laboratory studies, field studies are common for evaluating the impacts of oxidative stress in natural populations. Measurements of lipid and DNA damage combined with changes in the levels of antioxidant defenses can serve as a means of detecting exposure to xenobiotics that induce oxidative stress. However, the choice of end points in field studies of fish may be complicated by a history of exposure to xenobiotics causing oxidative stress. For instance, grey mullet (*Mugil* sp.) collected from an estuary polluted with metals, PAHs, PCBs, and pesticides demonstrated evidence of oxidative stress as indicated by oxidized glutathione redox status. However, these fish did not show elevated MDA levels while showing elevated activities of antioxidant enzymes (GPx, SOD, catalase, GR). It is possible that an adaptive response had occurred and that repair of lipid peroxidation may have taken place (144).

Because the response of the organism may be xenobiotic and tissue specific, it is also important to examine several end points. Otto and Moon (145) compared brown bullhead (*Ameriurus nebulosus*) that were collected from a system polluted with PCBs to bullhead collected from a relatively nonpolluted aquatic system. Fish from the polluted site had a 22-fold increase in PCB concentrations in white muscle compared to fish from the nonpolluted site. Cytosolic SOD activity was increased in the kidney of fish from the polluted site. Conversely, catalase activity in the kidney,

GPx activity in the red and white muscle, and total glutathione in the liver, kidney, and white muscle were decreased relative to fish from the nonpolluted site. Studies such as these advocate the use of a group of end points when evaluating oxidative stress in feral organisms.

Oxidative stress and disease. The link between oxidative stress and disease is also of interest to oxidative stress toxicology. Cancer is one disease for which fish may be especially well suited to serve as model organisms, and the appeal of using fish as models for studying the molecular basis of carcinogensis is growing. The benefits of using rainbow trout in addition to rodent models when evaluating environmental carcinogesis have been discussed by Bailey et. al. (146,147). Some of these benefits include low rearing costs that enable quantitative studies with as many as 10,000 fish as well as well-described tumor pathology. *Xiphophorus* fish models have been used to study the roles of genetics and UV radiation in melanoma tumorigenesis (148–151). Studies of oncogenes and tumor suppressor genes have also been conducted in fish. For instance, two *ras* genes are expressed in rainbow trout liver, and both contain a high degree of homology to the human *ras* genes. The predicted amino acid sequence of trout *ras-1* suggests a highly conserved protein and suggests that the function of the ras p21 protein is the same in both higher and lower vertebrates (152). Similarly, the tumor suppressor gene *p53* has been cloned in Japanese medaka (*Oryzias latipes*) (153) and rainbow trout (154) and shown to be strongly conserved in functional domains critical to tumor suppressor function in other vertebrates, including humans. Furthermore, Winn et. al. (155) have created transgenic medaka and mummichog (*Fundulus heteroclitus*) that contain multiple copies of the bacteriophage Phi X174am3cs70 that will enable comparisons of mutations in the same target gene sequence across species following xenobiotic exposure.

Studies that pertain specifically to the role of oxidative stress in cancer have also been conducted in fish. For instance, comparative studies between fish species have been done that evaluate changes in antioxidant defenses that may be linked to disease etiology. Channel catfish, which seldom express pollutant-mediated neoplasms, and brown bullhead, which often exhibit neoplasms in contaminated waters, differ greatly in their glutathione-dependent defense systems. Channel catfish have higher levels of hepatic GSH and γ-glutamylcysteine synthase activity, while bullhead have more hepatic GSSG and increased GPx activity (34). In a comparative study with menadione (a quinone) as the toxicant, channel

catfish exhibited a more rapid induction of total glutathione than did bullhead, likely due to the increased hepatic γ-glutamylcysteine synthase activity. Furthermore, when challenged with an organic peroxide (*tert*-butyl hydroperoxide), hepatic GSH was depleted in bullhead but not in channel catfish. This difference in GSH depletion was attributed to the differences in glutathione peroxidase and glutathione reductase activities between species (34). A different study of the same two fish species exposed to β-naphthoflavone showed that catfish exhibited significantly higher hepatic catalase and GR activities as well as total glutathione content, whereas bullhead exhibited higher hepatic GPx activity. Catfish showed higher levels of menadione-mediated cytosolic $\cdot O_2^-$ and H_2O_2 production, whereas bullhead demonstrated higher rates of $\cdot O_2^-$ and H_2O_2 production in microsomes. Liver homogenate from bullhead was also less effective at inhibiting Fe^{3+}-ascorbate-mediated production of 8-OHdG (156). The results from these two studies suggest that differences in antioxidant poise may result in differential abilities to defend against certain types of oxidative stress and may account for the difference in susceptibility to cancer between these two species (34,156).

Other studies have linked oxidative DNA damage to neoplasms in fish. As mentioned previously, the FapyGua lesion has been found in the DNA of neoplastic hepatic lesions in English sole (124), and hepatic DNA damage, in the form of both 8-OH adducts of guanine and adenine as well as FapyGua, has been reported in a population of English sole contaminated with PAHs and PCBs that also show a high incidence of liver cancer (125). The comparative studies of antioxidant poise in catfish, the studies on DNA damage in English sole, the demonstration of conserved oncogenes and tumor suppressor genes between mammals and fish, and advances in the use of fish as models for carcinogenicity suggest that markers of oxidative stress may be predictive of cancer risk in some instances and that fish may be useful in further elucidating the mechanisms of carcinogenesis.

Conclusions

Oxidative stress comprises an important aspect of toxicology and, consequently, has received increasing attention in recent years. During this time, research has moved from observing the effects of oxidative stress at the level of the organism to elucidating the mechanisms behind the responses and damages seen at the cellular and biochemical levels. Much of the research on oxidative stress has taken place in mammalian models as the

interest in oxidative stress has centered primarily around human health issues. However, increasing evidence suggests that oxidative stress is also of ecological significance, particularly in the aquatic environment which provides a sink for many pollutants capable of causing oxidative stress. Comparing the data available for both mammalian and piscine systems demonstrates that the toxic and adaptive mechanisms of oxidative stress are similar across species. This suggests that fish may serve as good biomonitoring tools, and, in addition to mammals, may provide useful models for further research into understanding oxidative stress.

There are several advantages to using piscine systems to study oxidative stress, such as the reduction in the number of mammals used in research, the potential for a reduction in the cost of animal maintenance, and the ability to increase the power of the experiment by increasing the number of organisms per study. Additionally, the developmental biology of some fish species, such as zebra fish (*Brachydanio rerio*) and mummichog, have been well studied (*157–159*). These species provide excellent models for studying the developmental aspects of oxidative stress toxicology, as the growing embryo can be observed throughout development. Moreover, most fish species are oviparous, enabling the evaluation of development in isolation as fertilization and development occur externally. With the recent advent of transgenic fish (*160–162*) comes the potential to perform knock-out experiments and evaluate how oxidative stress may influence gene expression in piscine models. Furthermore, the use of piscine models to study oxidative stress allows for the evaluation of environmental issues from both a human health and ecological perspective.
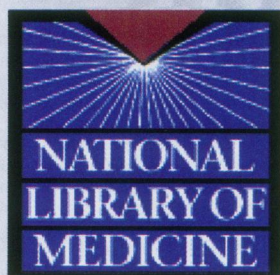
Many research opportunities still exist in oxidative stress toxicology and present the occasion to study oxidative stress in piscine systems. For example, little is known specifically about the repair processes that are utilized by cells to repair damage induced by oxidative stress. In addition to aiding in the understanding of how cells cope with oxidative damage, these processes may involve inducible enzymes or other responses that could serve as biomarkers of exposure or effect. Similarly, in light of evidence that oxidative stress can damage DNA and gametes, the impact of oxidative stress on processes such as reproduction and development warrants further study. Finally, there is still a need for research that definitively links oxidative stress and disease. Performing these studies in fish will increase our knowledge of oxidative stress mechanisms while expanding the ability to generalize the phenomenon of oxidative stress across species.

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